H I L G A R D I A

A Journal of Agricultural Science Published by the California Agricultural Experiment Station

VOL. 16 SEPTEMBER, 1944 No. 6

THE PICRIC ACID METHOD FOR DETERMINING MINUTE AMOUNTS OF HYDROCYANIC ACID IN FUMIGATED INSECTS[1, 2]

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INTRODUCTION

IN STUDIES ON the toxic effect of different concentrations of HCN (hydrocyanic acid) on fumigated insects, determinations of the amounts of the gas absorbed and retained are important. To secure these facts, an accurate method of determining amounts of HCN in the range of 0.005 to 0.200 mg is essential. Extensive preliminary investigations showed that the picric acid method had the greatest promise.

Picric acid in alkaline solution has been widely used as a reagent in colorimetry. It has been commonly used in determining soluble sugars in plant extracts and creatinine in animal fluids. In the procedures for determining reducing sugars, the reaction involves the reduction of the nitrophenol to aminophenol. In the presence of reducing sugars, picric acid (trinitrophenol) is reduced to picramic acid when heated in alkaline solution. The same basic reaction is involved in the determination of creatinine. According to the literature, a different reaction occurs between HCN and picric acid in alkaline solution.

Hlasiwetz (1859)[5] was the first to note the formation of isopurpuric acid according to the following equation:

$$\underset{\text{Picric acid}}{C_6H_3N_3O_7} + 3KCN + 3H_2O \rightarrow \underset{\text{Potassium isopurpurate}}{C_8H_4KN_5O_6} + CO_2 + NH_3 + 2KOH$$

Rosenthaler (1923), also, noted that blood-red isopurpuric acid is formed when picric acid and KCN are heated in alkaline solution. Some disagreement exists about the formation of isopurpuric acid. According to Chapman (1911), the reaction is identical with the reducing reaction between picric acid and reducing reagents, resulting in the formation of 2-amino-4,6-dinitrophenol. In the present experiments it was not determined which of the two reactions

[1] Received for publication August 4, 1943.

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[5] See "Literature Cited" at the end of the paper for complete data on citations, which are referred to in the text by author and date of publication.

occurs. Since either one results in a blood-red coloration, this determination is relatively unimportant.

For determining approximate amounts of HCN in cyanophoric plants, picric acid paper is often used : the crushed plant tissue is incubated in a closed container, from the top of which is suspended a strip of moist picric acid paper (Foy and Hyde, 1937). The HCN given off by the material reacts with the paper, changing the color to a reddish orange. This paper can be compared with a standard, or the color can be extracted from the strips and the resulting solution then compared with a standard solution. It is evident that such procedures are unsuited for quantitative determinations.

Many investigators have used alkaline picrate in HCN determinations (Adriano and Ynalvez, 1932; Nowosad and MacVicar, 1940; Hogg and Ahlgren, 1942). Waller (1910) estimated HCN in animal and plant tissues by distilling it from the sample, which had been acidified with tartaric acid, into a receiving tube containing a measured volume of alkaline picrate. After incubation for 24 hours at 30° C, the color was compared with standards containing known amounts of cyanide. Sullivan (1939), determining the HCN in white-clover plants, distilled the HCN into solutions of NaOH, from which aliquots were taken for the color reaction. He improved the method by measuring the color change with a photometer.

Even with Sullivan's refinement, however, the procedure was not sensitive enough for quantitative determination of such minute amounts as are encountered in fumigated insects. Detailed study of the entire procedure was therefore initiated to eliminate as many as possible of the variables affecting accuracy and to standardize the procedure so that concordant results could be obtained. The procedure given in the following section is based on this study. Some of the factors found to affect accuracy are discussed in a later section.

STANDARDIZED PROCEDURE

Apparatus and Reagents.—The colorimeter used in this work was an A. C. model, Fisher Electrophotometer. The absorption cells consisted of 23-ml cylindrical tubes. A green filter (5,250 angstrom units) proved to be most satisfactory and was used for obtaining the experimental data reported in this paper. A blue filter was first tried; but the instrument could not be balanced at the zero point against the blank solution of the reagents. When the blue filter was used, the colored solutions had to be compared with a distilled-water standard; that is, the most accurate part of the dial scale was used in measuring the difference between the distilled-water zero point and the reading for the blank solution. On the dial there is a calibration scale from which direct logarithmic readings can be obtained. This, designated as "scale A" on the graphs, simplifies the construction of a calibration curve, since the readings vary directly with the intensity of the color. The makers of the instrument advise that results are more accurate if readings fall between the zero point and a scale-A reading of 50.

The apparatus used in these experiments for distilling the HCN is shown in figure 1. It consisted of a small distilling flask (fig. 1, *B*), with a glass side arm that had an opening 0.6 mm in diameter. The mouth of the flask was fitted

with a ground-glass standard-taper stopper (fig. 1, *A*), through which passed a sealed-in aeration tube extending to within 5 mm of the bottom of the flask. This tube had a lower opening 0.3 mm in diameter. An opening of this size permitted sufficient air to pass through, kept the sample stirred better, and gave more accurate results than did a very fine capillary. Minute quantities of HCN (0.005 to 0.200 mg) could be distilled with this simple apparatus without loss due to rubber stoppers (Morris and Lilly, 1933).

The receiving tube (fig. 1, *C*) was a small test tube, 10 × 1 cm, with a short side arm to connect with the vacuum pump. The distillation flask and receiving tube were of the same size, except that the former had a small bulge blown into it. That small enlargement gave much smoother distillation and prevented the sample from jamming up around the aeration tube.

The reaction containers found most suitable for this determination were Pyrex test tubes graduated at 10 ml and 25 ml. Such tubes eliminate the necessity of transferring solutions to volumetric flasks to bring them all to constant volume. If desired, sugar-analysis tubes graduated at 12.5 ml and 25 ml can be substituted, provided they are used both in constructing the standard curve and in analyzing the sample. The importance of the reaction volume is explained later in this paper (p. 296).

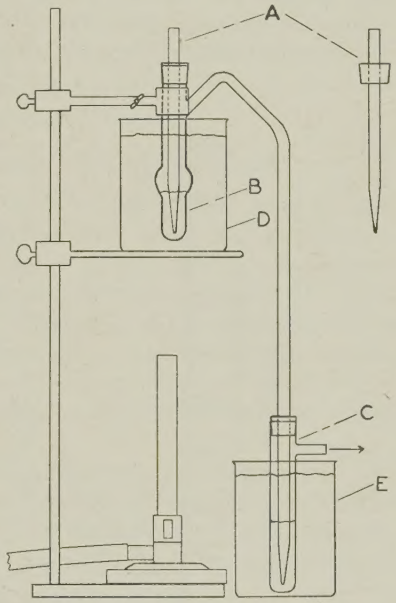

Fig. 1.—Diagram of apparatus used in distilling HCN from insect samples: *A*, aeration tube with standard-taper ground-glass stopper; *B*, distilling flask with connected glass side arm; *C*, receiving tube with outlet to suction; *D*, water bath; and *E*, ice bath.

The reagents used were as follows:

Picric acid solution, prepared by dissolving 10 grams of C.P. picric acid in distilled water and diluting to a final volume of 1 liter.

Sodium carbonate solution, prepared by dissolving 50 grams of the anhydrous salt in distilled water and diluting to a final volume of 1 liter.

Sulfuric acid solution, approximately 10 *N*, prepared by diluting 27.8 ml of concentrated H_2SO_4 to a 100-ml volume.

Sodium cyanide solution, prepared from the moisture-free salt. The solutions used in this work were equivalent to 0.1 mg of HCN per milliliter of solution. The exact amount of NaCN necessary for that concentration depends on the purity of the salt. If 100 per cent pure NaCN were available, 0.1814 gram of the salt, made up to a liter with distilled water, would give the desired concentration. Since most brands of NaCN are only 95 to 96 per cent pure, however, a correction must be made. The HCN content was checked accurately by titration with silver nitrate (Liebig method). A fresh NaCN solution had to be prepared at least once a week, because dilute solutions of the salt decom-

pose in the presence of air. In most instances one preparation will be enough, since the solution is necessary only in constructing the calibration curve.

Distillation.—The sample (100 to 200 mg of insects) was placed in the distillation flask and made to about 2 ml with water. In the receiving tube was placed 1 ml of the Na_2CO_3 reagent, diluted to 3 ml with distilled water to give more volume of alkali for absorption of the distillate.

The distillation unit was then connected and stoppered tightly, with just enough vacuum applied to the receiving tube to maintain a slow but steady flow of bubbles through the Na_2CO_3. One drop of 10 *N* H_2SO_4 was added to the cyanide sample through the aeration tube. The mixture was placed in a

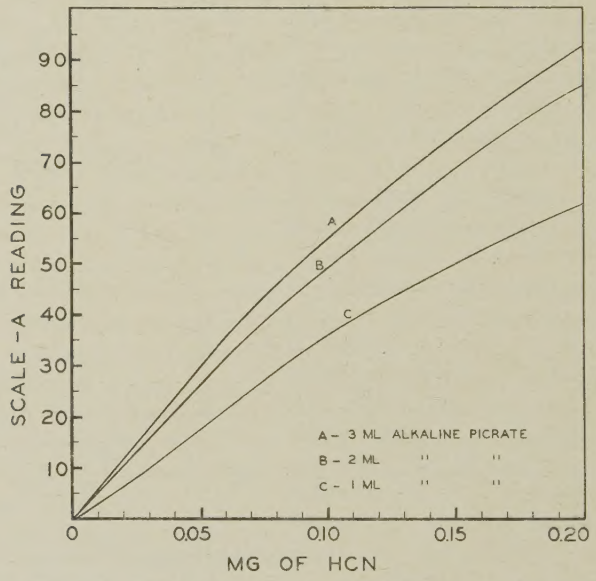

Fig. 2.—Calibration curves of HCN, showing effect of using different amounts of alkaline picrate.

water bath and maintained at the boiling point for 20 minutes. The receiving tube was immersed in an ice bath to keep the carbonate solution cold enough to trap all the HCN in the distillate.

Color Reaction.—After the sample had been distilled for 20 minutes, the distillate was carefully transferred to the reaction tube. The receiving tube and the delivery tube were washed carefully with water to remove any Na_2CO_3 remaining on them. This wash water was added to the distillate, the total volume of the distillate and washings in no case exceeding 9 ml. One milliliter of the picric acid solution was added to the distillate, and the volume was brought to 10 ml with distilled water, if necessary.

After its contents had been thoroughly mixed, the reaction tube was heated exactly 3 minutes in a boiling-water bath and allowed to stand 1½ to 2 hours before its volume was diluted to 25 ml. The tube was then placed in a constant-temperature bath (25° ± 1° C) for 10 minutes, after which its contents were transferred to an absorption cell and the colorimeter reading was taken. The

reference cell in the colorimeter contained a blank solution of the reagents. From this reading, the HCN present was calculated by means of a calibration curve.

Calibration Curve.—For construction of the calibration curve, various amounts of the standard NaCN solution were added to 2 ml of the alkaline picrate reagent, consisting of equal volumes of Na_2CO_3 and picric acid solutions mixed just before using. (The results are more consistent if the carbonate and the picric acid are kept separate until needed.) The cyanide and alkaline picrate mixture was diluted to 10 ml in the reaction tubes. For the color reaction these mixtures were treated exactly the same as the distillates described above. The scale-A readings for the standard mixtures were plotted against the concentration of HCN present (fig. 2).

FACTORS AFFECTING COLOR DEVELOPMENT

In colorimetric analyses the procedure must be exactly the same in each determination. For accurate results, definite rules must be adhered to. Although proposed for visual colorimetry, many of the precautions outlined by Dehn (1917) are even more important in photometry. Preliminary experiments on the alkaline picrate method showed that several important variables must be controlled. For that reason, factors such as reaction volume, length of heating period, time between heating and reading, dilution, and temperature at time of reading were carefully studied to determine how they affect the accuracy of the results.

Reagent Used for Receiving Distillate.—In previous experiments on this method, the HCN was distilled from the samples either into alkaline picrate reagent (Waller, 1910) or into NaOH (Sullivan, 1939). In the present experiments, both these methods proved unsatisfactory for determining minute amounts of HCN.

When the HCN is distilled directly into alkaline picrate, any volatile reducing substances present in the sample are also distilled into the reagent, and there is no longer any chance of separating the HCN from the impurities.

Experiments were performed to determine whether distillation into NaOH had any effect on color intensity. Alkaline picrate reagent was prepared by mixing equal volumes of the Na_2CO_3 and picric acid solutions described on page 296, and 2 ml of the mixture was put in a reaction tube. Varying amounts of 0.1 *N* NaOH or of 1.0 *N* Na_2CO_3 solution (in addition to the carbonate solution in the alkaline picrate reagent) were added, then 0.05 mg HCN. The volume was brought to 10 ml with distilled water, and the color intensity was determined as described on page 294. According to these data, shown graphically in figure 3, the presence of NaOH decreases the color intensity, whereas excess Na_2CO_3 has no effect at all. The reason, probably, is that the NaOH partially neutralizes the picric acid and causes a greater change in pH than does the carbonate.

According to the results in figure 3, Na_2CO_3 gave the greater promise of being a satisfactory receiving solution. A series of distillates was therefore compared with a series of standard mixtures. In one series, known volumes of the standard NaCN solution were distilled, and the HCN was received in a solution of 1 ml of the carbonate reagent diluted to 3 ml with distilled water.

The standard mixtures were prepared by adding the NaCN directly to alkaline picrate reagent. The amounts of HCN in the distillates and in the standard mixtures were determined by the procedure outlined on page 294. As numerous determinations showed, the variation in the amount of HCN determined in the distillate and in the standard-mixture series was no greater than the experimental variation among duplicate standard-mixture samples of the same concentration of HCN. In all cases, 100 per cent of the HCN was recovered on the distillates (table 1).

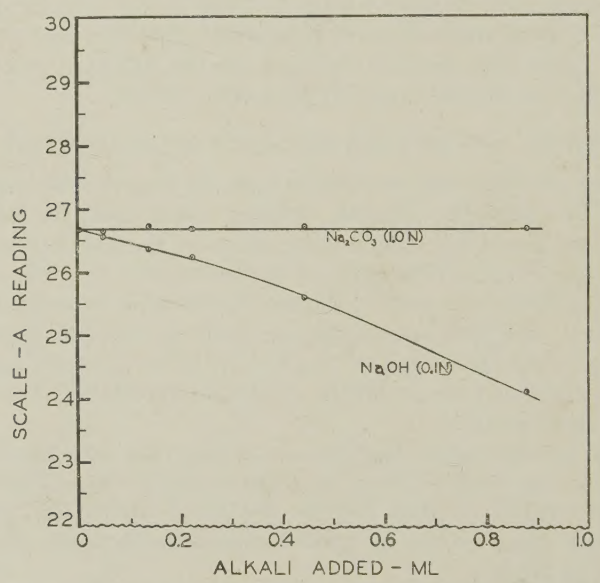

Fig. 3.—Effect of different amounts of Na_2CO_3 and NaOH on the color intensity of alkaline picrate with 0.05 mg HCN added.

Amounts of Na_2CO_3 and Picric Acid.—It is not necessary to use 2 ml of alkaline picrate reagent as described under "Standardized Procedure"; but the amount used in the standard mixtures for the calibration curve must be equivalent to the Na_2CO_3 and picric acid solutions used in analyzing the unknown samples. If a greater volume of alkaline picrate reagent is used, the slope of the curve increases (fig. 2, *A*). For work on fumigated insects a 2-ml volume of the alkaline picrate was very convenient. This amount, made up of equal volumes of the carbonate and picric acid solutions, gives a reagent of the same concentration as that used by Sullivan (1939) and by Hogg and Ahlgren (1942).

Reaction Volume.—The HCN and alkaline picrate mixture, when heated in the water bath, must be rigidly controlled at a constant volume for all determinations. Any change in the concentration of the reacting substances causes a similar change in the concentration of the colored compound produced. In effect, therefore, an increase in volume of solvent is a decrease in concentration of solute. As figure 4 shows, the intensity of color for a given amount of HCN, reacted with a 2-ml sample of alkaline picrate, decreases rapidly with an increase in reaction volume.

If desired, the reaction volume can be made more or less than 10 ml, the volume that was used in this problem in order to obtain color differentiation from small quantities of HCN. If the volume is increased, however, it is advisable to increase the alkaline picrate in each mixture. If, for example, the reaction volume is to be 25 ml, 5 ml of the reagent should be used. When a small reaction volume—10 ml—is used, the final dilution eliminates any error resulting from unequal evaporation during heating.

Length of Heating Period.—In the first experimental work, reaction mixtures were heated in boiling water for 5 minutes, according to the procedure

TABLE 1

RELATIVE AMOUNTS OF HCN DETERMINED BY THE PICRIC ACID METHOD ON STANDARD MIXTURES AND ON DISTILLATES FROM KNOWN QUANTITIES OF HCN

HCN added	Standard mixture,* scale-A reading	Distillate†	
		Scale-A reading	HCN recovered
mg			*mg*
0.005	2.4	2.4	0.005
.010	5.1	5.2	.010
.020	10.7	10.7	.020
.030	16.1	16.0	.030
.040	21.4	21.4	.040
.050	26.8	26.7	.050
.060	31.5	31.6	.060
.080	40.6	40.7	.080
.100	49.3	49.4	.100
.150	69.4	69.6	.150
0.200	86.3	86.3	0.200

* Standard mixtures prepared by direct addition of NaCN to 2 ml of alkaline picrate were diluted to 10 ml, heated 3 minutes, allowed to stand for 2 hours, diluted to a final volume of 25 ml, and then read at 25° C.

† Distillates were prepared by distilling the NaCN into 1 ml Na_2CO_3 (50 grams per liter), according to the procedure described. (Text, p. 294.)

of Sullivan (1939) and of Hogg and Ahlgren (1942). Investigations showed, however, that excess heat diminishes the color development. Maximum color intensity was obtained when the heating time was 2½ to 3 minutes. Heating for a longer period appears to decompose the color products formed in the first 2 or 3 minutes. All values reported in this paper were obtained from solutions heated 3 minutes.

Time between Heating and Reading.—As the heated solution cools to room temperature, the color intensity begins to increase. This development extends over several hours. One must therefore read both the standard and the unknown mixtures in the photometer at about the same length of time after heating. In this way the error from unequal color development is eliminated. Two hours gave more consistent results than a shorter period.

Dilution When HCN Concentration Is High.—The amounts of HCN in the insect samples tested were so minute that the photometer readings came within the recommended range (scale-A reading 0 to 50) with no further dilution than is indicated in the standardized procedure—a final volume of 25 ml, all the distillate being used.

If there is much more HCN in the distillate, dilution will be necessary to bring the readings within the desired range. Such dilution, however, may introduce errors. Willaman and Davison (1924), while investigating the picric acid method for determining sugars, found that dilution gave results 5 to 10 per cent high.

An experiment was therefore conducted to determine the point in the procedure at which the dilution can most safely be made. To test the effect of dilution after the reaction, a series of solutions was prepared, all having a reaction volume of 10 ml with 2 ml of alkaline picrate, but with the NaCN solution varied to give the equivalent of 0.05 to 0.50 mg of HCN. Duplicate

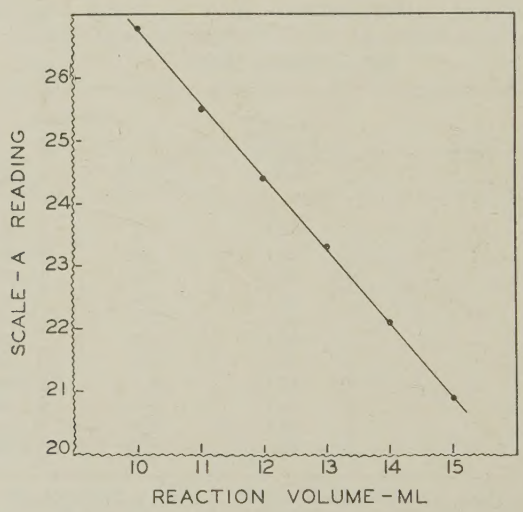

Fig. 4.—Effect of changes in the reaction volume on color development in 2 ml alkaline picrate with 0.05 mg HCN added.

samples were prepared. The solutions were heated 3 minutes, allowed to stand 2 hours, and then diluted to final volumes proportionate to the NaCN added, the volume used with 0.05 mg of HCN being 25 ml as in the standardized procedure. If the color formed were proportionate to the HCN added, all volumes should thus give the same reading as the 25-ml volume.

Table 2 indicates that this does not happen. The 50- and 100-ml volumes gave readings slightly higher than the 25-ml, but the differences are probably not significant. The 200- and 250-ml volumes, on the other hand, gave readings considerably lower. A possible explanation is that a comparatively large amount of HCN will not give a full depth of color because of the change in the equilibrium ratio between the amount of HCN and the amount of picrate reagent present. Though the experiments do not indicate the underlying reasons, Beer's law apparently does not hold for these dilutions.

The only accurate method of bringing high HCN concentrations within the scale range, then, is to dilute the distillate before adding the picric acid. Often one will have to run a trial-and-error series of dilutions to determine the approximate range. Dilution of the distillate affords an opportunity to take

aliquots for replicate determinations, a check not possible with the low HCN concentrations in the tested insects.

Temperature at Time of Reading.—Although variations in temperature cause only slight variations in the readings, solutions should be brought to the same temperature before the readings are made. In the HCN and alkaline picrate system, a temperature rise causes an increase in the reading. The latter is small, however, in comparison with the temperature increase. When 0.05 mg HCN was present, an elevation of 15 degrees Centigrade resulted in an increase of 1 unit on the scale-A reading. A water bath of any arbitrary

TABLE 2

EFFECT OF DILUTION ON THE COLOR INTENSITY OF THE HCN-ALKALINE-PICRATE SOLUTION*

Amount of HCN	Final volume of picrate solution	Scale-A reading			HCN recovered, average of two samples	
		First sample	Second sample	Average	Amount	Percentage
mg	*ml*				*mg*	*per cent*
0.05	25	26.8	26.8	26.8	0.050	100.0
.10	50	27.4	27.7	27.6	.103	103.0
.20	100	27.3	27.4	27.4	.204	102.0
.40	200	24.8	24.8	24.8	.368	92.0
0.50	250	23.0	23.1	23.1	0.423	84.6

* All solutions had reaction volume of 10 ml with 2 ml of picrate; they were heated 3 minutes and allowed to stand 2 hours, then diluted to the various volumes, and read at 25° C with green filter.

temperature (preferably 18° to 25° C) may be used to bring the solutions to the same temperature, allowing a deviation of ± 1 degree. All readings in this report were made at 24° to 25°.

DISCUSSION

The chief criticism of this method is the nonspecificity of the reaction. Any volatile reducing substances possessed by the material being distilled are usually carried over with the steam into the receiving flask, and subsequently react with the alkaline picrate. Some insects—for example, the confused flour beetle, *Tribolium confusum* duV.—have been found to give off volatile reducing substances during the distillation. Preliminary distillations on the unfumigated insects are therefore essential, to determine whether substances that might react with the picric acid are present in the distillate. Most of the insects experimented upon were free of such substances. Partial success has been achieved in eliminating these reducing impurities, when present, by redistilling the distillate at a lower temperature.

With this method, the possible range in concentration for the determinations is 0.005 to 0.200 mg HCN; but the most suitable range is 0.01 to 0.10 mg HCN. Use of the smaller range results in readings between 0 and 50 on the A scale of the colorimeter. One can vary the range somewhat, if necessary, by changing the volume of alkaline picrate used for each determination.

This method has been used in determining HCN from fumigated scale insects and walnut-husk-fly pupae. The experimental data on these insects are published in the accompanying paper (Lindgren and Sinclair, 1944).

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RELATION OF MORTALITY TO AMOUNTS OF HYDROCYANIC ACID RECOVERED FROM FUMIGATED RESISTANT AND NONRESISTANT CITRUS SCALE INSECTS

D. L. LINDGREN AND WALTON B. SINCLAIR

RELATION OF MORTALITY TO AMOUNTS OF HYDROCYANIC ACID RECOVERED FROM FUMIGATED RESISTANT AND NONRESISTANT CITRUS SCALE INSECTS[1, 2]

D. L. LINDGREN[3] and WALTON B. SINCLAIR[4]

Resistance to fumigation with hydrocyanic acid gas (HCN) in the red scale, *Aonidiella aurantii* (Mask.), was first noted in 1914; and resistance in black scale, *Saissetia oleae* (Bern.), in 1915 (Quayle, 1938).[5] Sufficient experimental data have accumulated to establish definitely the existence of two strains or races of red scale in the citrus areas of California: one race that is susceptible and another that is tolerant to HCN fumigation. These two races have been reared in the laboratory for the past seven years, under identical conditions in separate insectproof rooms, through an average of nine generations per year, or a total of some sixty generations; and they are still maintaining the original differential in susceptibility to HCN. As Dickson (1941) demonstrated, this difference in susceptibility depends on a single gene or group of closely linked genes in the X chromosome and therefore is sex-linked. The black scale has been less studied, because of the difficulties involved in rearing it from generation to generation; but, according to recent work by Lindgren and Dickson (1943), the resistant and the nonresistant black scale differ as much in their tolerance to HCN as do the two races of red scale, or even more.

In an effort to determine the basis for the difference in the reaction of the resistant and nonresistant races of scale insects to HCN fumigation, two lines of investigation were followed in the present experiments. In the first, the fumigation experiments, an attempt was made to evaluate separately the influence of HCN concentration and of exposure on the mortality of the two races of red scale. In the second, the sorption experiments, an attempt was made to correlate the mortality of fumigated resistant and nonresistant races of both red and black scales with the amounts of HCN sorbed (as measured by the amounts recovered); the effects of varied dosages, exposures, and pretreatments on sorption and mortality were studied.

MATERIALS AND METHODS

In the experiments here reported, studies were made of resistant and nonresistant mature adult female red scale, reared under controlled laboratory conditions, in insectproof rooms. The variation in age of the insects was less than 24 hours, since the scale crawlers were transferred to grapefruits from the stock cultures several times daily.

In the fumigation experiments, two series were conducted: in one the con-

[1] Received for publication August 4, 1943.
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[5] See "Literature Cited" at the end of the paper for complete data on citations, which are referred to in the text by author and date of publication.

centration of HCN was held constant and the time of exposure varied, whereas in the other the exposure time was held constant and the HCN concentration was varied. The fumigations were conducted in a 100-cubic-foot fumatorium at 75° F. Counts to determine mortality were made 2 weeks after treatment.

Since a method was available (Sinclair and Ramsey, 1944) for recovering small amounts of HCN accurately from fumigated insects, a series of experiments was set up to determine how much cyanide could be recovered from resistant and nonresistant red scale. Each insect was carefully removed from the fruit, with little or no injury; according to earlier observations, the adult red scale will survive and produce young for at least 36 hours after being taken from the host. The sample used in the sorption experiments consisted of 500 to 700 individuals. The insects were counted and weighed before fumigation. The average weight of a nonresistant specimen was 0.212 mg; of a resistant one, 0.214 mg. Two persons removed the insects from the fruit (this work took 30 to 45 minutes); and, to eliminate any personal factor, the two alternated in handling the resistant and nonresistant races. After being weighed, the insects were placed in the bottom of petri dishes; and the samples, one nonresistant and the other resistant, were fumigated in a 100-cubic-foot fumatorium at 75° F. The relative humidity varied from 45 to 60 per cent in the several experiments, no effect on sorption being noted within this range of humidities. Immediately after fumigation, the insects were placed in the distillation flasks, and the distillation process was begun. The two races of red scale always received identical treatments before fumigation, after fumigation, and throughout distillation.

FUMIGATION EXPERIMENTS

Some light might be thrown on the basis for the difference in HCN susceptibility of different races of scale insects, and hence on advisable commercial fumigation practice in various districts, if the effects of concentration and of exposure could be distinguished. Most experiments on resistant and nonresistant scale insects have been concerned with concentrations and exposures simulating those used in the field, little being done with high dosages and short exposures or low dosages and long exposures. In one of the few experiments designed to evaluate the effects of concentration and exposures separately, Moore (1936) found: "The concentration to which the resistant red scale in California is exposed is of double the value of the length of exposure in effecting a kill. . . . The concentration to which nonresistant red scale in California is exposed is of approximately equal value to the length of exposure in effecting a kill."

In comparing mortality curves of the two races of red scale, only the part that covers the mortality range between 15 per cent and 98 per cent is of interest. At either extreme of the typical mortality curve, the line flattens out: with high dosages and long exposures, a 99 to 100 per cent kill of both races will be obtained; with low dosages and short exposures there is no or very low mortality of both; and if either of these two factors has a zero value, the mortality will obviously be zero. But comparisons at the extremes would not justify the conclusion that the two races do not differ in susceptibility to HCN. Hence in the experiments reported in this paper, the values of concen-

tration and exposure chosen were such as could be expected to yield kills in the significant portions of the mortality curves.

In the first series of fumigations, the exposure was held constant at the very low value of 1 minute, and the concentration was varied from 4.0 mg to 9.6 mg per liter, values many times those used in commercial practice. The results were as follows:

Milligrams of HCN per liter	Per cent mortality of nonresistant race	Per cent mortality of resistant race
4.0	52	26
5.6	48	37
8.0	47	36
9.6	45	22

Although the two races showed differences in susceptibility to HCN, and all percentages were in the "significant" range, there was no increase in mortality of either race, even when the concentration was more than doubled. Judging from this result, with very short exposures only a limited amount of HCN is taken up by the insect.

In the second series, the exposure was increased to 10 minutes, a value high enough to reveal the effects of different concentrations. For the nonresistant race, concentration varied from 0.19 mg to 1.60 mg per liter, for the resistant from 0.4 mg to 3.9 mg, both ranges extending below and above that used in commercial practice (about 1.0 to 1.3 mg per liter).

In the third series, concentration was held constant at 0.4 mg per liter, and the exposure was varied from 2½ minutes to 25 minutes for the nonresistant scale and from 10 minutes to 90 minutes for the resistant.

The results for the second and third series are presented graphically in figures 1 and 2. The concentration-mortality and exposure-mortality curves in these figures have been converted to straight lines by Bliss's (1935) methods. Net mortality is plotted in probits on the vertical axis. The independent variable—concentration or exposure—is plotted on a logarithmic scale on the horizontal axis. In both figures, the lines are almost parallel, those for the nonresistant scale being slightly steeper than those for the resistant. These lines show clearly that a difference exists in the mortality of the two races—a difference affected by the concentration or the exposure.

This difference is further shown by the following data, which give the percentage increase in concentration and in exposure required to increase the mortality by 1 probit:

Race	Percentage increase in exposure required to increase kill by one probit	Percentage increase in concentration required to increase kill by one probit
Nonresistant	89	80
Resistant	109	100

According to these data the percentage increase in exposure required to increase the mortality by 1 probit is greater for the resistant race than for the nonresistant. In other words, the nonresistant race is the one more affected by a given exposure increment.

The data also show that the percentage increase in HCN concentration required to increase the kill by 1 probit is greater for the resistant red scale

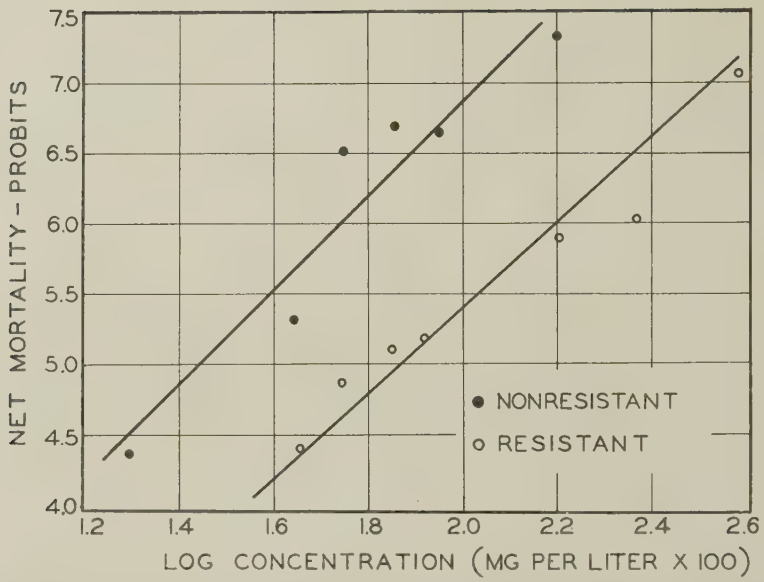

Fig. 1.—A comparison of the effect of different concentrations of HCN upon the mortality of mature adult nonresistant and resistant red scale. The exposure time was 10 minutes.

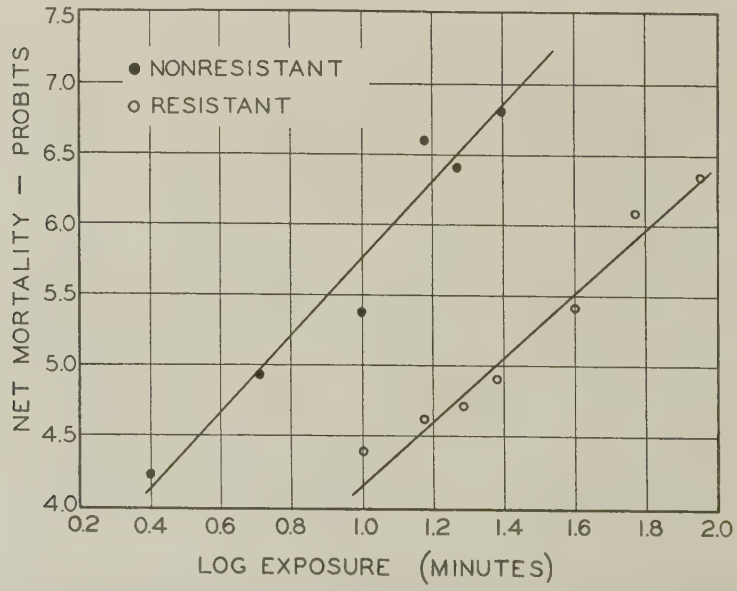

Fig. 2.—A comparison of the effect of different lengths of exposure upon the mortality of mature adult nonresistant and resistant red scale. The concentration of HCN was 0.4 mg per liter.

than for the others. Clearly, again, with the mature adults, the nonresistant race is the one more affected.

To sum up: at least under the conditions of these experiments, both HCN concentration and exposure to HCN are more important in effecting an increase in the mortality of the nonresistant than of the resistant red scale.

SORPTION EXPERIMENTS

RECOVERY OF HCN FROM RESISTANT AND NONRESISTANT RED SCALE

Methods for accurate determination of HCN and studies of factors affecting HCN recovery from citrus tissues have been published by Bartholomew and Raby (1935) and Bartholomew, Sinclair, and Janes (1939). In an attempt to correlate injury of citrus tissues with the amount of HCN recovered after fumigation, Bartholomew, Sinclair, and Lindgren (1942) reported: "The physiological condition of the tissues rather than environmental influences or the amount of HCN absorbed seems to determine whether they will or will not be injured by HCN after fumigation at night; injury after day fumigation appears to result from the effects of sunlight, which raises the temperature and influences the physiological condition of the tissues." This information immediately raises the question whether the differential in mortality of resistant and nonresistant red scale can be correlated with the amount of HCN absorbed by the two races.

Many theories regarding red-scale resistance to HCN have been published. Moore (1933) states: "With the data accumulated to date, it would appear that the difficulty of killing the resistant red scale was due to the difficulty of reaching the insect through its scale (waxy covering) rather than to any distinct immunity of the insect to hydrocyanic acid." But as Lindgren (1941) has shown, the same differential in resistance between the two races exists in the crawler, which has no waxy covering, as in the adult insect.

Haas (1934), studying the composition of red scale, found no relation "between the ability of the insects to resist fumigation and their organic or inorganic iron or (ash) phosphorus content." Since citrus scale insects contain rather large amounts of copper, he suggested that a reduced copper content may be related to fumigation resistance.

Quayle (1938) made no attempt to explain the mechanism of resistance to HCN, but did suggest some possible causes: "The question as to whether it is due to a difference in the waxy covering, or whether this is more tightly sealed to the surface in the case of the red scale, to a difference in respiratory rate, to a difference in nervous response, or to some other factor or factors, is not investigated."

Although Carpenter and Moore (1938) were not working with scale, their findings regarding the sorption of HCN by various other insects may be related to the problem of resistance within species. They concluded: "The amount of hydrocyanic acid sorbed varies with the species; those insects which are generally known to be difficult to kill were found to sorb smaller quantities of hydrocyanic acid than those species which are easily killed."

In the next paragraph are quoted observations made by Hardman and Craig (1941) in their studies on resistant and nonresistant red scale.

In each race the spiracles close within 3 to 5 minutes after the cyanide reaches them. In the resistant race the spiracles remain closed as long as HCN is present for at least 30 minutes. In the nonresistant race the spiracles remain closed for only about 1 minute and then open and death follows in a short time if the cyanide concentration is lethal. The resistant scale can survive a lethal concentration of cyanide for at least 30 minutes. . . . There seems no doubt but that the relative ability to maintain closure of the spiracles is sufficient to explain the difference in resistance to HCN of the two races.

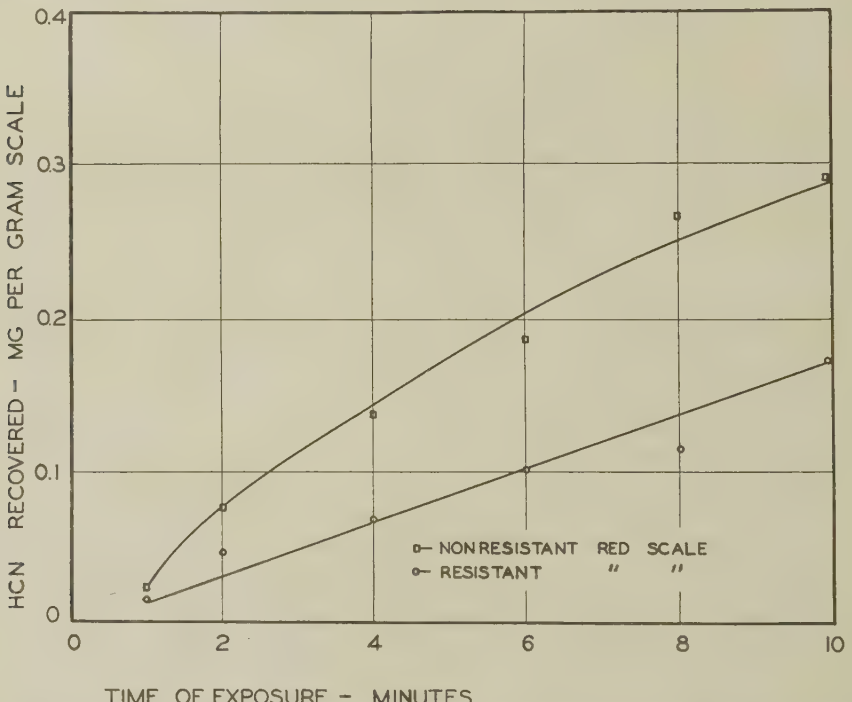

Fig. 3.—Recovery of HCN from resistant and nonresistant red scale. The fumigation concentration was 16 mg of HCN per liter, the temperature 75° F, and the relative humidity 45 to 60 per cent.

This conclusion is apparently based on the assumption that HCN does not penetrate the integument, but can obtain access to the scale tissues only through the spiracles.

Figure 3 graphically presents the amounts of HCN recovered from resistant and from nonresistant red scale fumigated at 75° F with a concentration of 16 mg of HCN per liter. As will be noted, the nonresistant scale sorbed much larger amounts of HCN than the resistant, at all exposures from 1 minute to 10 minutes. The points follow in line very well, considering that each exposure time is the result of a separate fumigation. At a 1-minute exposure, 50 per cent more HCN was recovered from the nonresistant scales than from the others; and at a 10-minute exposure 80 per cent more. These great differences indicate that the amount of HCN recovered must be related to the difference in kill of the two races.

[6] According to personal correspondence these authors, by subsequent experiments, have shown that "the integument is permeable to HCN since scale insects can be killed by HCN when it has no access to the spiracles."

The values reported in figures 3, 4, 5, and 6 designate the amounts of HCN recovered from the insect samples immediately after fumigation. They do not necessarily represent the total HCN sorbed during the fumigation period. With the method used in this study, the HCN recovered from a given insect sample would be equal to the total amount sorbed only if the body tissues of the insect were not capable of chemically reacting with the HCN. If, however, the body tissues of the insects are capable of chemically reacting with or fixing some of the HCN sorbed during a fumigation, then the amounts afterward recovered from the sample are proportionately reduced. The difference in the amounts recovered from the resistant and nonresistant scale may be due to the difference in the amounts fixed by the two races. But the quantity of HCN chemically changed or fixed by the tissues during fumigation is probably very small as compared with the total sorbed, so that the HCN recovered from the samples immediately after fumigation is believed to represent adequately the comparative sorption capacities of two samples fumigated under identical conditions. This theory agrees with the previously mentioned results obtained by Carpenter and Moore (1938) ; the latter used values representing the total sorption of HCN at a given gas pressure and including both the free HCN in the tissues and the HCN that may have been chemically combined.

In earlier experiments with dosage mortality, a concentration of 4.0 mg of HCN per liter and a 2½ minute exposure killed 99.6 per cent of the nonresistant scales, 91.3 per cent of the resistant. One can assume, accordingly, that at an exposure of 2 or 4 minutes and a concentration of 16 mg of HCN per liter all (or nearly all) of the two races would be killed. Then the differences in sorption of gas obtained (fig. 3) are due either to the first minute or two the insects (still alive) are exposed to the HCN, or else to something besides the mechanism of respiration. That the first of these two explanations is not the correct one is indicated by the trend of the curves: the curves tend to diverge as the exposure increases, and the amounts absorbed per gram of scale also increase with the exposure and approach a saturation value for a given concentration of HCN. If the difference in sorption cannot be explained by respiratory processes carried on while the insect is living, it may be due to some inhibitor or to some chemical reaction that takes place in the resistant scale and changes the HCN to some other compound. Plumb[7] suggests the following explanation: "May it not be that this ability to resist fumigation is based on the development of a counter-mechanism within the respiratory enzymes; so that inhibition of the enzyme by CN^- is blocked?" Although such a mechanism may be a factor in the resistance of red scale to HCN, the theory does not explain the entire picture, since it assumes that the HCN is present within the tissues of the resistant red scale but is prevented from acting. As shown by the experiments discussed above, there is an actual difference in the amounts of HCN recovered—a fact indicating that the HCN either does not get into the resistant scale so readily as into the nonresistant, or else is changed over when it does get in.

To determine whether this difference in HCN sorption is due to the first minute or two when the scales (still alive) are exposed to the HCN, several series of experiments were conducted: The scales were first killed by HCN,

[7] Plumb, George H., personal correspondence with H. J. Quayle, February 15, 1942.

lack of oxygen, or high temperature, with exposures previously determined to be lethal. The two races showed no difference in tolerance to high temperature or lack of oxygen. Scales killed in either of these ways were immediately removed from the hosts (grapefruits) and fumigated. Those killed by HCN were held for 5 hours before fumigation; 4 hours had been found by careful checking to be sufficient for complete dissipation of the sorbed HCN. Another series of tests (table 1, series 5) was conducted to determine how much HCN was sorbed by insects dead and completely dried; the interval after death was 19 days. A check series (no. 1, table 1) was run without pretreatment. In all series, scales were placed in the distillation flasks immediately after fumigation, and the HCN recovered. Table 1 gives the results.

TABLE 1

RECOVERY OF HCN FROM RESISTANT AND NONRESISTENT RED SCALE

(All samples fumigated at 16.0 mg HCN per liter for 10 minutes at 75° F and 45 to 60 per cent relative humidity)

Series no. and treatment before sorption fumigation	HCN recovered per gram of scale (fresh weight)	
	Resistant race	Nonresistant race
	mg	*mg*
1. Check: live scale, no pretreatment	0.171	0.302
2. Scale previously killed by lack of oxygen	.286	.321
3. Scale previously killed by high temperature	.184	.252
4. Scale killed by HCN fumigation 5 hours before sorption fumigation	.423	.301
5. Scale killed by HCN fumigation 19 days before sorption fumigation; completely dried out	0.120	0.121

As will be observed from this table, when the scales are killed by lack of oxygen or by high temperature, the resistant ones sorb less HCN than the nonresistant; but the difference is not so great as when the insects are alive. If the scales are killed by HCN 5 hours before the sorption test, the reverse is true: the resistant ones take up more HCN than the others. If the insects are dead and entirely dried, there is no difference in the amount of HCN absorbed.

From these observations one might deduce that the difference in recovery is due to some chemical change of HCN in the tissues of the scale. To explain the reversal obtained when the scales are fumigated and killed by the HCN before the recovery tests, one may infer some upset of the process whereby the resistant scales are enabled either to sorb less HCN than the nonresistant or else to change the HCN over to some other form. This theory would account for the resistant scales' taking up as much HCN as the nonresistant scales, but does not explain why after being killed beforehand with HCN they actually take up more. Because such differences are obtained in HCN sorption by dead resistant and nonresistant scales, and because the curves continue to diverge with increasing sorption up to saturation values (fig. 3) long after the scales are known to be dead, one may conclude that the differential in HCN sorption is not entirely due to the mechanism of respiration.

Apparently, therefore, under these experimental conditions, the sorption

of HCN by resistant and nonresistant red scale involves, also, such principles as rate of diffusion of the gas through the external and into the internal tissues, or the chemical reaction of bodily constituents with HCN so as to fix it and chemically change it into nontoxic substances. That such physical and chemical factors should be considered in the sorption of HCN by insects, especially in high concentrations, is essential for the elucidation of this problem.

In another series of experiments with resistant and nonresistant red scale, a 40-minute exposure was used with various concentrations of HCN. Figure

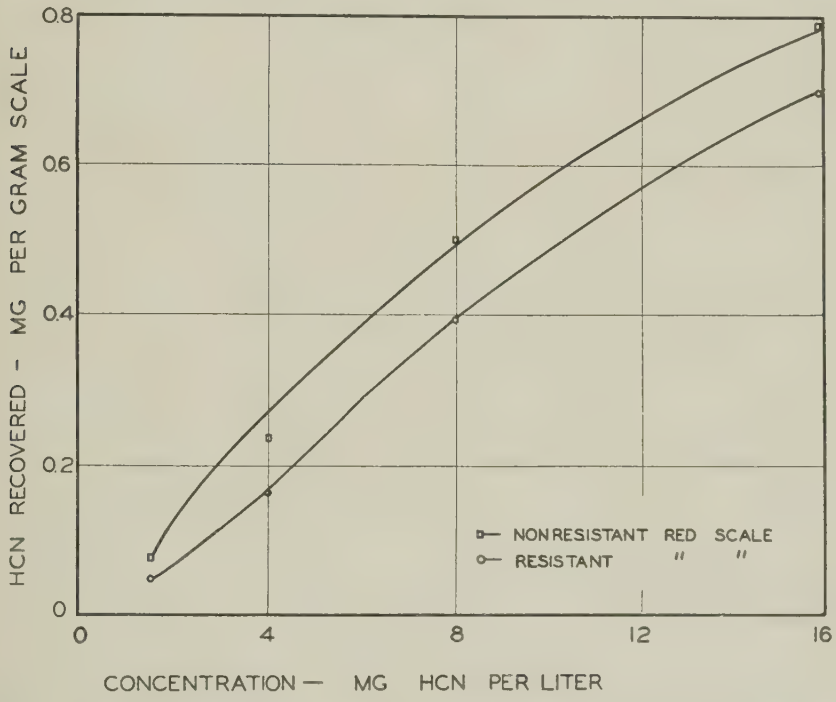

Fig. 4.—Recovery of HCN from resistant and nonresistant red scale. The exposure was 40 minutes, the temperature 75° F, and the relative humidity 45 to 60 per cent.

4 shows the results graphically. Again the nonresistant scale insects consistently take up more HCN than the others. The lowest concentration used in this series of experiments was 1.8 mg per liter, which is only slightly higher than that used in the field in commercial practice. At this concentration the nonresistant red scales sorbed 52 per cent more HCN than the resistant; and at 4 mg HCN per liter, 45 per cent more HCN.

In comparing the sorption curves (figs. 3 and 4) with the mortality curves (figs. 1 and 2), one immediately notes the similarity between them. Indications are that the amount of HCN recovered from the two races of red scale is directly related to the mortality. In like manner Carpenter and Moore (1938), working with various species of insects, concluded that "the ease of killing with hydrocyanic acid does not depend so much upon its actual toxicity, which is, of course, great for all forms of animal life, as it does upon how

readily the hydrocyanic acid is sorbed by the insects." Evidently, high sorption of HCN is associated with ease of killing. The degree of correlation cannot, however, be calculated from these and other published data; only after performing similar experiments upon many species of insects can one generalize. To illustrate, even when two species of insects sorb the same amount of HCN under identical conditions, the lethal dosage for each species may be very different.

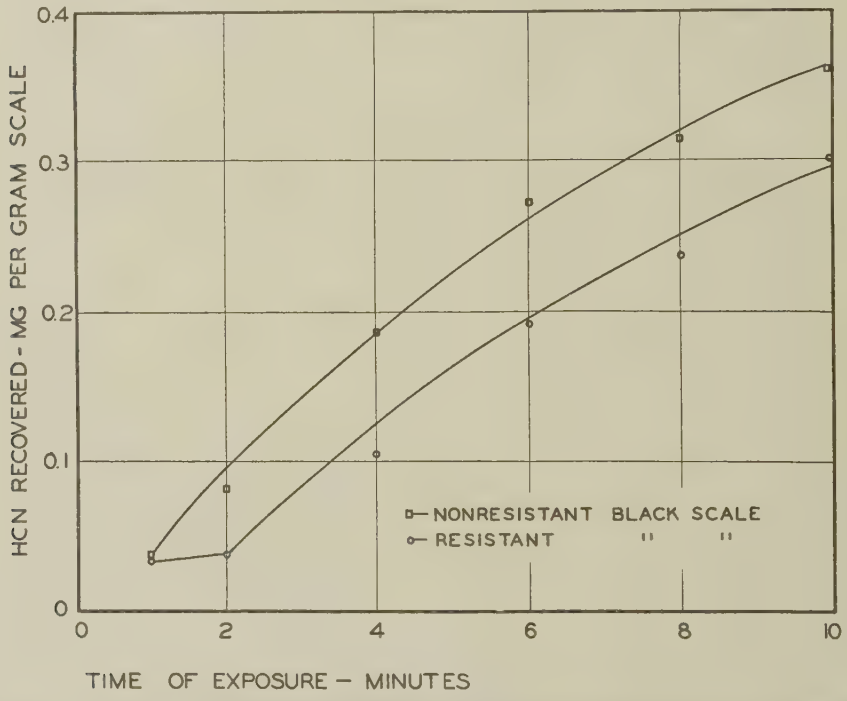

Fig. 5.—Recovery of HCN from resistant and nonresistant black scale. The fumigation concentration was 16 mg of HCN per liter, the temperature 75° F, and the relative humidity 45 to 60 per cent.

RECOVERY OF HCN FROM RESISTANT AND NONRESISTANT BLACK SCALE

In 1915 (Quayle, 1938) black scale, *Saissetia oleae* (Bern.), in the Charter Oak district of Los Angeles County, proved resistant to HCN. Lindgren and Dickson (1943) found differences in the tolerance to HCN of black scale reared in the laboratory under identical conditions.

At the time the sorption tests were being conducted, no black scales were being reared in the laboratory. Specimens were obtained in the field, however, from two groves—one grove known from previous tests (Lindgren and Dickson, 1943) to have resistant and the other nonresistant black scale. Conditions of the experiment were the same as for red scale. Figure 5 gives results of the sorption tests. At the 1-minute exposure there was no difference in the amount of HCN recovered from the two races of black scale; but in all the other exposures tried, the differences are great. Since the black scales obtained

in the field were large (4 to 6 mg each), perhaps a 1-minute exposure was not long enough to get much cyanide into the scale's body; the HCN recovered may be due to surface absorption. With black scale, as with red, more HCN is recovered from the nonresistant than the resistant race.

RECOVERY OF HCN FROM WALNUT-HUSK-FLY PUPAE

According to A. M. Boyce and R. B. Korsmeier,[8] pupae of the walnut husk fly, *Rhagoletis completa* Cress., are very difficult to kill with HCN; concentrations higher than 30 mg of HCN per liter, with 1-hour exposure, did not kill

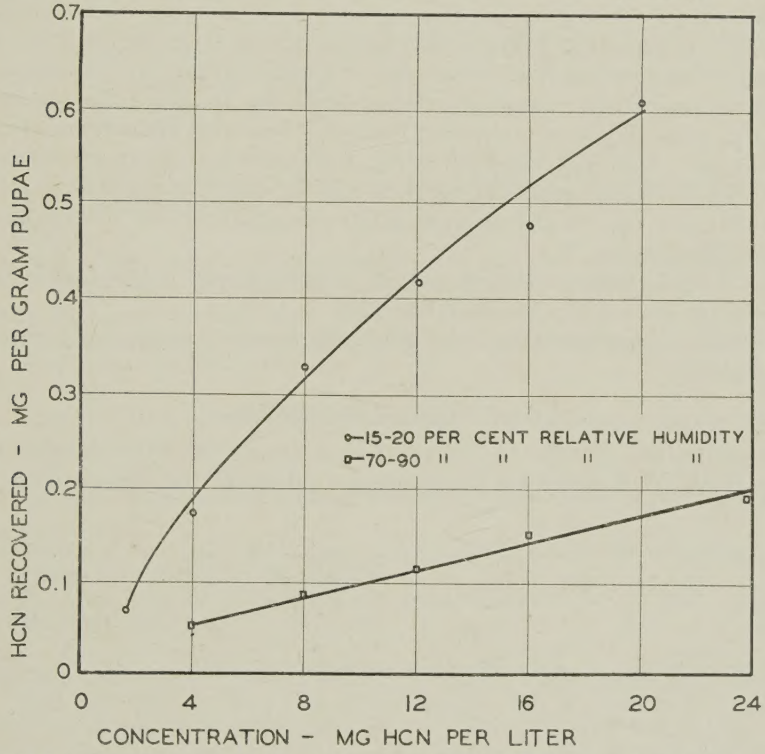

Fig. 6.—Effect of relative humidity on the recovery of HCN from walnut-husk-fly pupae. The time of exposure was 40 minutes, and the temperature 75° F.

100 per cent of the pupae. It was decided, accordingly, to determine how much HCN could be recovered from such pupae. The findings might have some bearing on the relation of HCN sorption to resistance in scale insects. Early in the work, this amount was found to vary with the relative humidity before and during the fumigation. Figure 6 shows the amount of HCN recovered from husk-fly pupae preconditioned and fumigated at a humidity of 15 to 20 per cent, and from another group preconditioned and fumigated at 70 to 90 per cent. Clearly, much more HCN is recovered from the former group. Comparing figure 4 with figure 6, one notes that less HCN is recovered from husk-fly pupae than from the resistant or nonresistant red scale. The red scale is also more susceptible to HCN than are the husk-fly pupae.

[8] Unpublished data on file at the University of California Citrus Experiment Station.

SUMMARY

Fumigation of nonresistant and resistant red scale indicates that the resistant race, with the various concentrations and exposures tried, is more difficult to kill than the nonresistant. Any added increment of either concentration or exposure has a greater effect on the kill of the nonresistant scale than on the others.

More HCN is recovered from fumigated nonresistant than from fumigated resistant red scale.

With red scale first killed by heat or lack of oxygen, and then fumigated, more HCN is recovered from the nonresistant than from the resistant race; but the difference is less than in insects fumigated without pretreatment.

With refumigated red scale previously killed by HCN fumigation but not dried up, more HCN is recovered from the resistant than from the nonresistant race. With red scale fumigated when dead and dried, equal amounts of HCN are recovered from the resistant and from the nonresistant race.

More HCN is recovered from fumigated nonresistant than from fumigated resistant black scale.

More HCN is recovered from walnut-husk-fly pupae if they are held and fumigated at low rather than high humidity.

More HCN is recovered from red scale than from walnut-husk-fly pupae, on a weight basis.

ACKNOWLEDGMENTS

The writers are grateful to Professor H. J. Quayle for his valuable counsel and to Mr. R. C. Ramsey for aid in certain phases of the experiments.

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